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Strategies for the Gas Phase Modification of Cationized Arginine via Ion/ion Reactions

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Abstract

The gas phase acetylation of cationized arginine residues is demonstrated here using ion/ion reactions with sulfosuccinimidyl acetate (sulfo-NHS acetate) anions. Previous reports have demonstrated the gas phase modification of uncharged primary amine (the N-terminus and ϵ -amino side chain of lysine) and uncharged guanidine (the arginine side chain) functionalities via sulfo-NHS ester chemistry. Herein, charge-saturated arginine-containing peptides that contain sodium ions as the charge carriers, such as $[\text{ac-ARAAARA}+2\text{Na}]^{2+}$, are shown to exhibit strong reactivity towards sulfo-NHS acetate whereas the protonated peptide analogues exhibit no such reactivity. This difference in reactivity is attributed to the lower sodium ion (as compared to proton) affinity of the arginine, which results in increased nucleophilicity of the cationized arginine guanidinium functionality. This increased nucleophilicity improves the arginine residue's reactivity towards sulfo-NHS esters and enhances the gas phase covalent modification pathway. No such dramatic increase in reactivity towards sulfo-NHS acetate has been observed upon sodium cationization of lysine amino acid residues, indicating that this behavior appears to be unique to arginine. The sodium cationization process is demonstrated in the condensed phase by simply spiking sodium chloride into the peptide sample solution and in the gas phase by a peptide-sodium cation exchange process with a sulfo-NHS acetate sodium-bound dimer cluster reagent. This methodology demonstrates several ways by which arginine can be covalently modified in the gas phase even when it is charged. Collisional activation of an acetylated arginine product can result in deguanidination of the residue, generating an ornithine. This gas phase ornithination exhibits similar site-specific fragmentation behavior to that observed with peptides ornithinated in solution and may represent a useful approach for inducing selective peptide cleavages.

INTRODUCTION

Site specific chemical derivatization approaches have long played important roles in the mass spectrometric analysis of peptides and proteins [1,2]. Historically, these types of bioconjugation reactions have been performed in the solution phase to improve ionization efficiency [3], provide for accurate relative quantification experiments [4,5], and facilitate primary [6,7] and three-dimensional structural characterization [8]. *N*-hydroxysuccinimide (NHS) esters have proven particularly useful as derivatizing reagents in tandem mass spectrometry (MS/MS or MSⁿ) experiments. They have been used, for example, to probe the reactivity of peptide and protein primary amine functionalities [8], to perform peptide and

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protein cross-linking studies for three-dimensional structural analysis [9,10,11], and to attach UV chromophores to peptides in order to facilitate photodissociation (PD) experiments [12]. Recently, it has been demonstrated that these traditionally off-line approaches can be performed on the millisecond time scale during the ionization process using reactive desorption electrospray ionization [13,14,15,16,17]. These types of targeted reaction chemistries provide the opportunity to obtain site-specific information about the analyte of interest within the context of a mass spectrometry experiment.

Over the past few years, our group has worked to develop methodologies by which these solution phase bioconjugation approaches can be executed in the gas phase using ion/ion reactions [18]. In addition to the short time scales over which ion/ion reactions can be performed (*i.e.*, 10s–100s of milliseconds), gas phase ion/ion bioconjugation approaches are attractive due to facile comparison between unmodified and modified analytes, a high degree of control over the precursor reactant identities via mass selection, the avoidance of complex product mixtures commonly generated in solution, and the ability to precisely control the number of covalent modifications. Recent demonstrations of these type of chemistries include the use of NHS ester derivatives in cross-linking [19,20] and covalent labeling studies [21,22,23], the use of 4-formyl-1,3-benzenedisulfonic acid (FBDSA) to tag and enhance the primary sequence coverage derived from collision induced dissociation (CID) experiments [24,25,26,27], and the use of N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide (CMC) in covalent labeling studies [28]. It has also been demonstrated that multiple derivatizations of this type may be performed “in parallel” (*i.e.*, during a single ion/ion encounter) at the cost of only a single analyte charge using cluster-type reagent ions composed of reactive reagent molecules as well as alkali metal counter ions [29].

A necessary condition for these types of gas phase chemistries to proceed is the formation of a long-lived ion/ion complex composed of ionic forms of both the analyte and reagent, at least one of which must be multiply charged in order to prevent neutralization. For example, in the reaction between a multiply protonated peptide analyte cation and a singly charged sulfo-NHS acetate reagent anion, long-lived complex formation is stabilized by an electrostatic interaction between the reagent sulfonate functionality and a protonation site on the peptide (*e.g.*, typically the N-terminus or side chain of a basic amino acid residue). Nucleophilic attack of the carbonyl carbon by a free basic residue on the peptide can then proceed, resulting in acetylation of the peptide and a loss of a neutral sulfo-NHS molecule upon collisional activation, a signature loss for this chemical reaction [21].

While there are strong parallels with solution phase behavior, it has been noted that the gas-phase reactions can show distinct behaviors, depending on the experimental ion/ion reaction conditions. For example, in contrast with the condensed phase behavior, it has been demonstrated that the arginine side chain can act as a suitable nucleophile for gas phase sulfo-NHS ester modification [23]. This distinct behavior is due to the high solution pK_a value of the arginine side chain guanidinium functionality (>12), which renders arginine side chains protonated under most reaction conditions. Protonation of the arginine side chain greatly reduces its nucleophilicity and renders it effectively unreactive with NHS esters. The highly basic solution pH values required to generate the free base of arginine readily hydrolyze the ester functionality of the NHS reagent. In the gas phase, on the other hand, pH is obviously an irrelevant consideration. Depending on the charge state and amino acid composition of the peptide sequence, arginine residues need not be protonated. Under these conditions, the high basicity of the free base form of arginine residues makes them superb nucleophiles for gas phase covalent modification via sulfo-NHS ester chemistry.

Under common positive ion electrospray ionization (ESI) conditions of peptides and proteins, the arginine side-chains are often fully protonated in the most abundant ions, which renders them unreactive with NHS esters. Suitable arginine reaction conditions to this point have been achieved through the use of low charge state peptide ions containing a number of arginine residues in excess of the number of peptidic charges and the use of charge-inversion style reactions where the analyte peptide cation is converted to an anion using a multiply deprotonated reagent [23,30]. Here, we demonstrate the selective covalent modification of cationized arginine using a sulfo-NHS acetate reagent anion. While the protonation of arginine renders it unreactive towards sulfo-NHS type reagents, cationization by sodium ions maintains arginine reactivity. This reactivity is demonstrated to occur with cationized arginine-containing peptides that have been generated in solution (*i.e.*, by adding NaCl to the peptide solution prior to ionization) and that have been generated in the gas phase (*i.e.*, through a gas phase proton-sodium cation exchange process). Additionally, the reactivity of a sodiated basic site is demonstrated to be unique to arginine and is only minimally observed with lysine residues.

EXPERIMENTAL

Materials

Sulfo-NHS acetate was purchased from PierceNet (Thermo Fisher Scientific Inc., Rockford, IL). Sodium chloride, ammonium bicarbonate, sodium tetraborate, acetyl chloride, and hydrazine hydrate were purchased from Sigma-Aldrich (St. Louis, MO). Methanol and acetic anhydride were purchased from Mallinckrodt (Phillipsburg, NJ). The peptides AKAAKA, ARAAKA, AKAAARA, and ARAAARA were custom-synthesized by NeoBioSci (Cambridge, MA). Stock peptide solutions at concentrations of ~1 mg/mL were prepared in water and then diluted up to 10-fold in 50/50 (v/v) water/methanol before use. Solution phase sodiated peptides were prepared by adding ~10 μ L of a ~5.5 mM NaCl solution to ~100 μ L of stock peptide solution.

Acetylation, Methyl Esterification, and Arginine Hydrazinolysis

Solution phase acetylation of N-termini, methyl esterification of C-termini, and arginine hydrazinolysis were all performed using established procedures. Briefly, acetylation was performed by first dissolving ~0.2 mg of peptide in 20 μ L of a 50 mM ammonium bicarbonate solution (with lysine-containing peptides, a borate buffer was used to minimize acetylation of the lysine side chain). After addition of 50 μ L of a 3:1 MeOH:acetic anhydride solution to the peptide solution, the reaction was allowed to proceed for ~1 hour at room temperature. The product was then dried using a Centrivap Concentrator (Labconco, Kansas City, MO) and reconstituted in 50–100 μ L of water. Methyl esterification was performed by first adding 40 μ L of acetyl chloride dropwise to 250 μ L of cold, dry methanol. After 5 minutes, ~0.5 mg of peptide was dissolved in 100 μ L of this solution. The reaction was then allowed to proceed for ~2 hours at room temperature, after which the product was dried in the concentrator and reconstituted in 50–100 μ L of water. Arginine hydrazinolysis (*i.e.*, ornithination) was performed by adding 50 μ L of hydrazine to 50 μ L of a 100 mM peptide solution. The reaction was then allowed to proceed for 2 hours in a 60°C water bath.

Mass Spectrometry

All experiments were performed on a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Concord, ON Canada) previously modified for ion/ion reactions [31]. Briefly, peptide cations and reagent anions were sequentially ionized using alternatively pulsed nanoelectrospray ionization (nESI) [32,33]. When studying solution phase-sodiated peptides, harsh declustering interface conditions were used to maximize

sodiated peptide ion signal. Following ionization, precursor ions were alternately isolated using the Q1 mass filter prior to injection into the q2 reaction cell. Following a defined mutual storage ion/ion reaction period (typically 500–1500 milliseconds), the resulting product ions were transferred to the Q3 linear ion trap where MS^n and mass analysis via mass-selective axial ejection were performed [34].

RESULTS AND DISCUSSION

Ion/ion Reactivity of Peptides Sodiated in Solution

It has recently been demonstrated that sulfo-NHS ester reagents can be used to modify the free base of arginine residues in the gas phase via ion/ion reactions [23]. While gas phase sulfo-NHS ester modification of the free base of arginine is much more facile than condensed phase modification of arginine, the requirement of a free base arginine residue can be a serious constraint on the range of peptide ions that can be modified under positive ESI conditions. A simple model peptide ac-ARAAARA is used here to illustrate this point (Figure 1). The N-terminal amine of this peptide was acetylated to prevent its modification, leaving the two arginines as the primary basic sites in the peptide sequence. An ion/ion reaction between the doubly protonated peptide, $[ac-ARAAARA+2H]^{2+}$, and singly deprotonated sulfo-NHS acetate, $[sulfoNHSacetate-H]^-$, which is denoted in the Figures as X, results in charge-reduced complex formation stabilized by an interaction between the sulfonate group on the reagent and a charge site on the peptide (Figure 1a). Collision induced dissociation of this long-lived ion/ion complex exhibits exclusive loss of the neutral sulfo-NHS acetate reagent ($-X_H$), resulting from proton transfer from the peptide to the reagent (Figure 1b). No signature loss of sulfo-NHS, which is indicative of covalent modification, is observed in this case, as would be expected when both basic residues of the peptide precursor are protonated. If, however, the excess charges of the precursor peptide are in the form of sodium ions (prepared by simply spiking the peptide solution with sodium chloride prior to ionization), the observed reactivity of the peptide system is remarkably different. An ion/ion reaction between the doubly sodiated peptide, $[ac-ARAAARA+2Na]^{2+}$, and the same sulfo-NHS acetate monoanion again results in complex formation (Figure 1c). CID of this complex, however, displays dominant covalent bond formation as is evident by the signature loss of neutral sulfo-NHS, resulting in acetylation of an α -nitrogen on one of the arginine residues (Figure 1d). In this instance, the neutral loss is comprised of the sulfo-NHS anion and a sodium counter cation (*i.e.*, $sulfo-NHS_{Na}$). We also note that the efficiency of initial ion/ion complex formation, relative to cation transfer, is quite different for the two peptide systems and seems to be dependent upon the nature of the cationizing agent. That is, the competition between complex formation and cation transfer (e.g., the formation of $[ac-ARAAARA+2H+X]^+$ and $[ac-ARAAARA+H]^+$ in Figure 1a versus the formation of $[ac-ARAAARA+2Na+X]^+$ and $[ac-ARAAARA+Na]^+$ in Figure 1c) favors complex formation with excess protons more so than with excess sodium ions. A similar tendency was noted for all peptide systems discussed here (data not shown). In the case of the protonated peptide, the key stabilizing interaction in the ion/ion complex is the $-OSO_3^- \cdots H^+ \cdots NH-$ interaction whereas the analogous interaction in the sodiated peptide is the $-OSO_3^- \cdots Na^+ \cdots NH-$ interaction. Given the much lower sodium ion affinities relative to proton affinities (Table 1), the depth of the well associated with the $-OSO_3^- \cdots Na^+ \cdots NH-$ interaction is expected to be significantly lower than that for the $-OSO_3^- \cdots H^+ \cdots NH-$ interaction. As a result, a large fraction of the ion/ion reaction intermediate complexes formed from the multiply protonated peptides survive. In the case of the sodiated peptides, a higher fraction of the initially formed complexes fall apart before a covalent reaction can take place. For those that do survive, a large fraction undergo covalent reaction (see Figure 1d).

We also examined the influence of sodium cationization on the reactivity of lysine residues. The reactivity of the unprotonated ϵ -amino side chain of lysine towards sulfo-NHS ester reagents, both in solution and in the gas phase, has been well documented [21,22]. As expected, CID of an ion/ion complex generated via a reaction between doubly protonated ac-AKAAAKA and the sulfo-NHS acetate anion results solely in loss of the neutral intact reagent, as depicted by a H/X_H ratio of 0 (Figure 2a). Here, a diamond (H/Na) is used to denote signature loss of neutral sulfo-NHS, either with a proton or sodium counter-ion, resulting in covalent modification and $X_{H/Na}$ is used to denote neutral loss of the intact reagent resulting in charge reduction of the peptide. Changing the precursor ion type from a doubly protonated ion to a doubly sodiated ion results in a measureable but small degree of covalent modification ($H/Na/X_{H/Na} < 0.2$). The corresponding comparison for ac-ARAAARA was $H/X_H = 0$ for the doubly protonated peptide and $H/Na/X_{H/Na} > 21$ for the doubly sodiated peptide. Sodium cationization clearly has a lower impact on the reactivity of lysine than it does on the reactivity of arginine. The mixed cation version of the arginine containing peptide, $[ac-ARAAARA+H+Na]^{2+}$, exhibits substantially greater reactivity ($H/Na/X_{H/Na} > 2$) compared to either the doubly protonated analogue ($H/X_H = 0$) or the doubly sodiated lysine containing system (see above).

In contrast to the relatively high reactivity of $[ac-ARAAARA+H+Na]^{2+}$, the mixed cation doubly charged versions of the two hetero-basic residue peptides, ac-AKAAARA and ac-ARAAAKA, are only modestly reactive ($H/Na/X_{H/Na} \approx 0.22$ each). When the hetero-basic peptides are doubly sodiated, substantial increases in reactivity are observed ($H/Na/X_{H/Na} > 20$ for ac-AKAAARA and $H/Na/X_{H/Na} > 0.6$ for ac-ARAAAKA). As discussed further below, the differences in proton and sodium ion affinities between the lysine and arginine side chains likely underlie the major change in reactivity in going from the mixed cation ions to doubly sodiated ac-AKAAARA and ac-ARAAAKA peptides. For the $[M+H+Na]^{2+}$ forms of these peptides, the thermodynamically favored cation partitioning is for the proton to associate with the arginine side-chain leaving the sodium cation to interact with the lysine residue. As has been shown, neither protonation of arginine nor sodiation of lysine would be expected to yield facile covalent modification using sulfo-NHS ester chemistry.

Interestingly, the reactive behaviors of the doubly sodiated forms of the two isomeric peptides are significantly different; $[ac-AKAAARA+2Na]^{2+}$ shows over an order of magnitude more covalent modification versus intact reagent loss when compared to $[ac-ARAAAKA+2Na]^{2+}$. While large $H/Na/X_{H/Na}$ ratios can be particularly sensitive to small variations in the $X_{H/Na}$ loss channel, the ratios here represent a significant disparity in reactivities. The difference in reactivities reflect what appears to be differences in the interactions of the basic amino acid side chains in the two isomeric forms of the peptide. The behaviors of the methyl esterified versions of the isomeric peptides provide compelling evidence for the importance of interactions with the C-terminus of the peptide (Figure 2b). Methyl esterifying both of these peptides gives $H/Na/X_{H/Na}$ ratios much more comparable to one another ($H/Na/X_{H/Na} \approx 3.1$ for ac-AKAAARA-OMe and $H/Na/X_{H/Na} \approx 10.6$ for ac-ARAAAKA-OMe). The C-terminus apparently enhances the reactivity of the $[ac-AKAAARA+2Na]^{2+}$ ion ($H/Na/X_{H/Na} > 20$) whereas it apparently diminishes the reactivity of the $[ac-ARAAAKA+2Na]^{2+}$ ion ($H/Na/X_{H/Na} \approx 0.6$). It has previously been proposed that an intramolecular interaction between the carboxyl-terminus and protonated arginine exists when the arginine residue is located near the C-terminal end of the peptide [38]. More specifically, spectroscopic studies indicate that sodium cationized arginine exists in a zwitterionic state in which the protonated arginine side chain donates one hydrogen bond to the N-terminus and the sodium ion is bicoordinated with the C-terminal carboxylate group whereas protonated arginine existed only in a nonzwitterionic form. In those reports, methyl esterification of the C-terminus disrupted the zwitterionic structure of the sodium cationized arginine [39,40]. Considering the lower ion affinity of the arginine side chain for sodium as

compared to a proton, a salt bridge structure such as that mentioned above likely enhances the nucleophilicity of the arginine guanidinium functionality, thereby making it more available for covalent modification. The arginine residue is likely too far from the C-terminus for this phenomenon to occur in the ac-ARAAKA peptide model. Methyl esterifying both of these peptides eliminates this enhanced reactivity (Figure 2b) and gives $H/Na/X_{H/Na}$ ratios much more similar to one another (though some degree of sodium cation charge solvation with backbone carbonyls likely still exists). Methyl esterification also increased the reactivity of the sodium containing ac-ARAAARA ions (see Figure 2). It is beyond the scope of this report to explore details of all of the intramolecular interactions that can affect the $H/Na/X_{H/Na}$ ratios. However, it is clear that intramolecular acid-base interactions, such as those involving the C-terminus, can play important roles in mediating the reactivity of arginine in peptides cationized with sodium ions.

Ion/ion Reactivity of Peptides Sodiated in the Gas Phase

Recently, it has been demonstrated that cluster-type reagent ions composed of n number of reactive sulfo-NHS ester reagent anions and $n-1$ alkali metal counter cations (resulting in a net cluster charge of -1) can be used to perform multiple covalent modifications in a single ion/ion encounter (given that the peptide analyte has a suitable number of free base reactive sites available for modification) [29]. This allows for multiple gas phase covalent modifications at the 'cost' of only a single analyte charge. In a similar manner, it could be envisioned that a cluster-type reagent could be used to transfer a metal counter cation, in this case a sodium ion, to the peptide in exchange for an excess peptide proton. By such a cation exchange process [41], an unreactive multiply protonated arginine-containing peptide (where any arginines present are protonated) could be converted into a reactive peptide in which at least one of the arginine residues is sodiated. The peptide ac-ARAAARA is used to demonstrate this methodology (Figure 3). An ion/ion reaction between the doubly protonated peptide, $[ac-ARAAARA+2H]^{2+}$, and a sodium-bound anion dimer of sulfo-NHS acetate, $[2X_{Na}]^{-}$, produces a long-lived ion/ion complex, $[ac-ARAAARA+2H+2X_{Na}]^{+}$. As there are no sites available for covalent modification, CID of this complex results in neutral loss of the intact sulfo-NHS acetate reagent (Figure 3a). Intact reagent neutral losses with either a proton, X_H , or a sodium counter ion, X_{Na} , represent competing dissociation pathways in this experiment (the other ions in Figure 3a are from consecutive dissociation from one of these two initial pathways). In this instance, the sodium counter ions originate from the reagent cluster while the proton counter ions originate from the peptide. The relative abundances of reagent neutral losses with either a proton or sodium counter ion are rationalized on the basis of the relative proton and sodium ion affinities of sulfate and arginine (Table 1). As the difference between the sulfate and arginine sodium ion affinities (73 kcal/mol) is larger than the difference between the sulfate and arginine proton affinities (69 kcal/mol), the more thermodynamically favorable cation partitioning is for the sulfate to bind a sodium ion and an arginine to bind a proton, which is consistent with the much larger abundance of X_{Na} loss as compared to X_H loss in Figure 3a).

In the case of the X_{Na} loss, the residual ion has the form $[ac-ARAAARA+2H+X]^{+}$. As there are no sodium cations in this complex, CID of this ion results only in further intact neutral loss ($-X_{Na}-X_H$) and does not result in covalent modification (Figure 3b). Conversely for the X_H loss of Figure 3a, the residual ion does have a sodium ion present and takes the form $[ac-ARAAARA+H+Na+X]^{+}$. Collisional activation of this ion results in abundant covalent modification (represented as $-X_H-sulfoNHS_{Na}$ and $-X_H-sulfoNHS_H$ in Figure 3c). The relative abundances of these secondary neutral losses with either a proton or sodium counter ion are once again consistent with the relative proton and sodium ion affinities of sulfate and arginine. The behavior observed here is fully consistent with the experiments described

above that used arginine-containing peptides sodiated in solution. The difference here is the introduction of a sodium ion into the complex via the reagent anion.

An ion/ion reaction between $[\text{ac-AKAAAKA}+2\text{H}]^{2+}$ and the same sulfo-NHS acetate reagent dimer cluster produces a similar long lived complex, $[\text{ac-AKAAAKA}+2\text{H}+2\text{X}_{\text{Na}}]^+$. Similar to the diarginyl peptide (Figure 3a), there are no sites on the peptide available for reaction and CID of this complex produces initial neutral loss of an intact reagent (Figure 4a). Here, neutral loss with a proton, $-\text{X}_{\text{H}}$, is favored over neutral loss with a sodium counter ion, $-\text{X}_{\text{Na}}$. Considering that the difference between the sulfate and lysine sodium ion affinities (76 kcal/mol) is smaller than the difference between the two proton affinities (82 kcal/mol), it would be expected for the sulfate functionality to preferentially bind a proton. As before, the other ions in Figure 4a are from consecutive dissociation of one of these two initial pathways. Collisional activation of the initial X_{Na} loss product (which is the ion of the form $[\text{ac-AKAAAKA}+2\text{H}+\text{X}]^+$), once again results only in further intact reagent loss (Figure 4b). Collisional activation of the initial X_{H} loss product (which is the ion of the form $[\text{ac-AKAAAKA}+\text{H}+\text{Na}+\text{X}]^+$), largely shows further intact reagent neutral loss (Figure 4c). Though there is a small amount of covalent modification evident in Figure 4c, indicated by the $-\text{X}_{\text{H}}\text{-sulfoNHS}_{\text{Na}}$ and $-\text{X}_{\text{H}}\text{-sulfoNHS}_{\text{H}}$ ions, this pathway is much smaller than the intact reagent loss pathway, indicated by the $-\text{X}_{\text{H}}\text{-X}_{\text{Na}}$ and $-\text{X}_{\text{H}}\text{-X}_{\text{H}}$ ions. The covalent modification pathway noted here with ac-AKAAAKA is also much less prominent than the covalent modification pathway noted in the analogous ac-ARAAARA experiment (Figure 3c).

Gas Phase Ornithination

The gas phase dissociative behavior of acylated arginine has recently been characterized using sulfo-NHS esters. Depending on experimental conditions (*e.g.*, the location of the arginine within the polypeptide sequence, the identity of the acylation group, *etc.*), several distinct dissociative pathways have been observed [30]. Included amongst these are deguanidination of the modified arginine residue induced by proton abstraction of the acyl guanidine π -nitrogen by the acyl guanidine π -nitrogen (Scheme 1, which is referred to as 'Path 1' in ref. 30), resulting in the formation of an ornithine residue. In cases where the acyl group contains β -hydrogens, loss of the modification from the arginine side chain via formation of a 6-membered ring involving the acyl group from the modification and the guanidinium group from the arginine (Scheme 2, which is referred to as 'Path 3' in ref. 30) leads to regeneration of the unmodified peptide. This behavior can be observed in the collisional activation of gas phase arginine-acetylated ac-ARAAARA, denoted as $[\text{ac-ARAAARA}+ \text{H}]^+$ (where a diamond, $\blacklozenge$, indicates acetylation of an arginine residue) (Figure 5a). In this case, the $[\text{ac-ARAAARA}+ \text{H}]^+$ precursor was formed via the gas phase cation exchange and subsequent covalent modification process described above and is simply the $-\text{X}_{\text{H}}\text{-sulfoNHS}_{\text{Na}}$ ion in Figure 3c. Figure 5a shows several fragmentation pathways, including what may be a 'Path 3' loss of the arginine acetylation (-42 Da). Previous studies have demonstrated that the loss of the N-terminal acetylation (which would also be a 42 Da loss) does not contribute significantly to the 42 Da loss observed in Figure 5a [30]. Additionally, loss of the guanidinium functionality from the other, unmodified arginine side chain in the peptide likely does not contribute to a large degree because it is charge-bearing, stabilizing it towards side chain fragmentation. Other fragment ions consistent with covalent modification of an arginine (*e.g.*, b_6) and small neutral losses are also observed. Included amongst these is a 'Path 1' loss of 84 Da from the $[\text{ac-ARAAARA}+ \text{H}]^+$ precursor ion. This loss is caused by proton migration within the acyl guanidine functionality and results in deguanidination of the modified arginine to generate an ornithine (denoted by the red 'O' in Figure 5a and the $[\text{M}-(\text{H}+\text{HN}=\text{C}=\text{NH})+\text{H}]^+$ precursor ion in Figure 5b).

It has recently been reported that the ornithination of peptides can induce site-specific cleavages upon collisional activation (*i.e.*, 'the ornithine effect') [42]. In those instances, a 6-membered lactam formed via a nucleophilic attack of the unprotonated α -amine of the ornithine on the peptide backbone carbonyl carbon results in cleavage of the amide bond C-terminal to the ornithine residue, generating a lactam-terminated b ion and a corresponding y ion (Scheme 3). This process is demonstrated using the ornithinated (red 'O') product generated in Figure 5a. CID of this ion, formally $[M+H-\text{HN}=\text{C}=\text{NH}]^+$, results in abundant y_5 and b_6-42 ion production (Figure 5b). The b_6-42 ion is simply the b_6 ion which has had one arginine residue converted to an ornithine. This ion and the y_5 ion can both be presumed to arise from the ornithine-facilitated cleavages noted above. Other ions consistent with the ornithination of one of the arginine residues are also present, including the b_3-42 ion. It should be noted that there is likely an isomeric mixture which makes up the $[M+H-\text{HN}=\text{C}=\text{NH}]^+$ precursor ion population: some in which the arginine closer to the N-terminus has been ornithinated and some in which the arginine closer to the C-terminus has been ornithinated (indicated by R/O nomenclature used in the peptide ladder inset in Figure 5b). The y_6 ion is not consistent with ornithination of an arginine residue and is most likely indicative of a minor precursor ion population which, in part, consists of loss of the N-terminal acetylation. The observed fragmentation behavior for this gas phase ornithinated product is distinctly different from that of the unmodified, singly protonated peptide, $[\text{ac-ARAAARA}+H]^+$ (Figure 5c). Compared to the unmodified peptide in Figure 5c, the gas phase ornithinated peptide in Figure 5b displays abundant y_5 and y_4 fragment ions and no b_6 fragment ion. The presence of the b_6-42 ion in Figure 5c is consistent with the neutral loss of an arginine guanidinium functionality (*i.e.*, conversion to an ornithine residue) following backbone fragmentation and is a commonly observed fragment of neutral arginine [43]. However, this pathway is not indicative of loss of the guanidinium from the precursor peptide (*i.e.*, $[\text{ac-ARAAARA}+H-42]^+$) followed by ornithine-facilitated cleavage; rather, it most likely occurs via guanidinium loss from the b_6 fragment. In any case, as the newly formed ornithine here is already the C-terminal residue, the b_6-42 ornithine product cannot facilitate the ornithine-facilitated selective cleavage process. The fragmentation behavior of the gas phase product (Figure 5b) is instead remarkably similar to that of a singly ornithinated ac-ARAAARA peptide (one arginine of which has been converted to an ornithine) prepared in solution (Figure 5d). The differences in relative abundances of the ornithine-facilitated cleavages between Figure 5b and 5d (*e.g.*, y_5) may be due to differences in preferential modification of either the N-terminal or C-terminal arginine between the gas phase and solution phase processes.

CONCLUSIONS

The gas phase reactivity of sodium cationized arginine residues in polypeptide analytes has been characterized via ion/ion reactions with sulfo-NHS acetate anionic reagents. Arginine-containing peptides that contain sodium ions as the charge bearing particles exhibit strong reactivity towards sulfo-NHS acetate whereas the protonated peptide analogues exhibit no such reactivity. This difference in reactivity is likely due to the lower ion affinity of the arginine for a sodium ion as compared to a proton, increasing the nucleophilicity of the arginine guanidinium functionality and thus making it available for covalent reaction with a sulfo-NHS ester. Sodium cationization as it relates to increased sulfo-NHS ester reactivity does not have a pronounced effect with lysine amino acid residues and appears to be unique to arginine. This sodium cationization can be performed either in solution by adding a sodium salt to the peptide sample or in the gas phase through a peptide-sodium cation exchange process using a sulfo-NHS acetate sodium-bound dimer cluster-type reagent and demonstrates several methods by which charged arginine can be covalently modified in the gas phase. Collisional activation of the gas phase-acetylated arginine product can result in loss of the modification along with the arginine guanidinium side chain functionality,

effectively forming an ornithine amino acid residue. This gas phase ornithinated product exhibits similar site-specific fragmentation behavior to that observed with ornithinated peptides generated in solution and is likely due to the favorable formation of a six-membered lactam ring C-terminal to the ornithine residue (*i.e.* 'the ornithine effect'). This process may represent a useful approach for inducing selective peptide cleavages.

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Highlights

- Sodium cationized arginine-containing peptides are reactive with sulfo-NHS acetate
- Protonated peptide analogues exhibit no such gas phase reactivity
- Sodium cationization does not enhance lysine amino acid reactivity
- Sodium cationization can be done in solution or in the gas phase via cation exchange
- CID of acetylated arginine can form ornithine to induce site-specific fragmentation

Figure 1.

An ion/ion reaction between the sulfo-NHS acetate monoanion (denoted as X) and (a) $[\text{acARAAARA}+2\text{H}]^{2+}$ forms a long-lived ion/ion complex. (b) CID of the doubly protonated peptide/reagent ion/ion complex solely produces neutral loss of the intact sulfo-NHS acetate reagent. (c) An ion/ion reaction between the same sulfo-NHS acetate monoanion and $[\text{acARAAARA}+2\text{Na}]^{2+}$ also forms a long-lived ion/ion complex, but in this instance (d) CID of the doubly sodiated peptide/reagent ion/ion complex largely produces signature neutral loss of sulfo-NHS, indicating the covalent acetylation of an arginine residue. The lightning bolt (⚡) is used to denote the ion subjected to collisional activation. The asterisk (*) in 1c indicates a contaminant from sample preparation unrelated to the peptide.

Figure 2.

Ratio of covalent modification (Y) to intact reagent loss (X) as a function of ion type ($[M+2H]^{2+}$, $[M+H+Na]^{2+}$, and $[M+2Na]^{2+}$) observed for CID of ion/ion complexes composed of a sulfo-NHS acetate monoanion and a doubly charged (a) ac-AKAAAKA, ac-AKAAARA, ac-ARAAAKA, or ac-ARAAARA or (b) ac-AKAAARA-OMe, ac-ARAAAKA-OMe, and ac-ARAAARA-OMe. Proton and sodium counter ion loss channels associated with either loss of neutral sulfo-NHS or neutral sulfo-NHS acetate have been summed into a single channel, Y or X respectively.

Figure 3.

(a) CID of a complex, $[\text{ac-ARAAARA}+2\text{H}+2\text{X}_{\text{Na}}]^+$, generated via an ion/ion reaction between $[\text{ac-ARAAARA}+2\text{H}]^{2+}$ and $[2\text{X}_{\text{Na}}]^-$ shows initial neutral loss of X_{Na} and X_{H} . (b) Collisional activation of the initial X_{Na} loss results solely in further intact reagent neutral loss whereas (c) collisional activation of the initial X_{H} loss results largely in covalent modification.

Figure 4.

(a) CID of a complex, $[\text{ac-AKAAAKA}+2\text{H}+2\text{XNa}]^+$, generated via an ion/ion reaction between $[\text{ac-AKAAAKA}+2\text{H}]^{2+}$ and $[2\text{XNa}]^-$ shows initial neutral loss of X_{Na} and X_{H} . (b) Collisional activation of the initial X_{Na} loss results solely in further intact reagent neutral loss and (c) collisional activation of the initial X_{H} loss results in minimal covalent modification.

Figure 5.

CID of (a) gas phase arginine-acetylated ac-ARAAARA, (b) the ornithinated product (red 'O,' which is an 84 Da loss from the precursor ion) generated from 5a, (c) unmodified, singly protonated ac-ARAAARA, and (d) ac-ARAAARA singly ornithinated in solution. The diamond () is used to denote the presence of a gas phase acetyl modification.

Scheme 1.

Deguanidination of an acylated arginine residue results in the formation of an ornithine residue via a dissociation mechanism previously termed 'Path 1.'

Scheme 2.

Loss of the acyl modification from the guanine functionality results in the formation an unmodified arginine residue via a dissociation mechanism previously termed 'Path 3.'

Scheme 3.

A nucleophilic attack of the carbonyl carbon by the ϵ -amine of the ornithine side chain results in 6-membered lactam formation, generating a lactam-terminated b-type fragment and the corresponding y-type fragment in a dissociation process previously termed 'the ornithine effect.'

Table 1

Estimated proton and sodium affinities of lysine and arginine [35]. For the sulfate moiety, the proton affinity of hydrogen sulfate [36] and the sodium affinity of methyl sulfate were used [37].

Functional Group	Proton Affinity (kcal/mol)	Sodium Affinity (kcal/mol)
Sulfate	320	127
Lysine	238	51
Arginine	251	54